Antimicrobial Peptides Increase Tolerance to Oxidant Stress in *Drosophila melanogaster**□S

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It is well appreciated that reactive oxygen species (ROS) are deleterious to mammals, including humans, especially when generated in abnormally large quantities from cellular metabolism. Whereas the mechanisms leading to the production of ROS are rather well delineated, the mechanisms underlying tissue susceptibility or tolerance to oxidant stress remain elusive. Through an experimental selection over many generations, we have previously generated *Drosophila melanogaster* flies that tolerate tremendous oxidant stress and have shown that the family of antimicrobial peptides (AMPs) is over-represented in these tolerant flies. Furthermore, we have also demonstrated that overexpression of even one AMP at a time (*e.g. Diptericin*) allows wild-type flies to survive much better in hyperoxia. In this study, we used a number of experimental approaches to investigate the potential mechanisms underlying hyperoxia tolerance in flies with AMP overexpression. We demonstrate that flies with *Diptericin* overexpression resist oxidative stress by increasing antioxidant enzyme activities and preventing an increase in ROS levels after hyperoxia. Depleting the GSH pool using buthionine sulfoximine limits fly survival, thus confirming that enhanced survival observed in these flies is related to improved redox homeostasis. We conclude that 1) AMPs play an important role in tolerance to oxidant stress, 2) overexpression of *Diptericin* changes the cellular redox balance between oxidant and antioxidant, and 3) this change in redox balance plays an important role in survival in hyperoxia.

Oxygen is essential for aerobic life. However, except for the beneficial role in wound healing, too little or too much oxygen can induce morbidity and mortality (1, 2). Mammalian aging and numerous diseases such as neurodegenerative diseases and chronic inflammatory diseases, as well as injury to the heart, lungs, retina, brain, and other organs due to ischemia and reperfusion states, result, by and large, from oxidant injury (3–6). High O_2-induced oxidant injury could occur in cells in every organ, especially in the lungs, retina, heart, and brain (7, 8). Prolonged exposure to high O_2 generates excessive reactive oxygen species (ROS),[2] induces cell death and oxidative stress responses, affects the immune response and DNA integrity, and modulates cell growth (5, 9–11).

Drosophila melanogaster has similar O_2 response pathways as mammals, and research on flies has enhanced our understanding of oxidant stress (12–14). In the past, through an experimental selection design over many generations, we have successfully generated *D. melanogaster* flies that tolerate tremendously high O_2-induced oxidative stress. Microarray analysis has revealed that the family of antimicrobial peptides (AMPs) is over-represented among the up-regulated genes in these tolerant flies. We have further demonstrated that overexpression of even one AMP gene at a time (*e.g. Diptericin* (*Dpt*)) allows wild-type flies to survive much better in hyperoxia (15). To our knowledge, this is the first study to show that *Dpt* has a protective role against oxidative stress.

In this study, we used a number of experimental approaches to investigate the potential mechanisms of hyperoxia tolerance and to understand how *Dpt* overexpression protects flies from oxidative injury. We demonstrate that flies with *Dpt* overexpression resist oxidative stress by increasing antioxidant enzyme activities, including superoxide dismutase (SOD), catalase, and GST, and by preventing an increase in ROS level under hyperoxia. By feeding buthionine sulfoximine (BSO), a chemical that depletes the GSH pool and inhibits antioxidant enzyme activities, we further confirm that enhanced survival observed in these flies is related to improved redox homeostasis.

EXPERIMENTAL PROCEDURES

Fly Stocks and Culture—da-Gal4 was obtained from the Bloomington Stock Center. *UAS-AMP* flies (*w; imd, UAS-AMP; spz^{rm7}, UAS-AMP/TM6C*) were generously provided by Dr. Bruno Lemaitre (Global Health Institute, Lausanne, Switzerland). All stocks were maintained on standard cornmeal-agar medium.

*Hyperoxia Chambers—*Experimental chambers (26 × 16 × 16 cm) were specially designed and supplied with 90% O_2 balanced with N_2. Humidity was maintained by passing the gas through water prior to entry into the chambers. The flow speed was monitored with a 565 glass tube flow meter (Concoa, Virginia Beach, VA), and the O_2 level within the chamber was periodically tested with a Diamond General 733 Clark-

2 The abbreviations used are: ROS, reactive oxygen species; AMP, antimicrobial peptide; SOD, superoxide dismutase; BSO, buthionine sulfoximine.

style electrode (Diamond General Development Corp., Ann Arbor, MI).

Hyperoxia Treatments—Embryos were cultured in normoxia for 48 h and then transferred to the 90% O_2 chamber. After ~3 weeks in culture, the percent eclosion (adult emergence) was determined by calculating the ratio of the number of empty pupae to the number of total pupae in each culture vial. Experiments were repeated at least three times for each line. The 3–5-day-old adult flies were exposed to 90% O_2, and the survival rate was scored every day. At least 200 flies in each group were used.

RNA Extraction and Microarray Analysis—Total RNA was extracted as described previously (15). Affymetrix *Drosophila* Genome 2.0 arrays (Affymetrix, Santa Clara, CA) were used, and probe labeling, array hybridization, and image scanning were performed following the standard protocol according to the manufacturer's instructions. The resulting data files from the Affymetrix scanner were background-subtracted and normalized using Robust Multi-array Average software. All comparisons of mRNA expression levels between the groups were performed using VAMPIRE (variance-modeled posterior inference with regional exponentials). Genes were identified as differentially expressed if they showed a fold-change of at least 1.5 with a *p* value < 0.05. The list of differently expressed genes was subjected to a subsequent post-analysis task such as MAPPFinder2 and STRING 8.3 software to find the main biologic processes associated with the experimental system and potential network interactions among genes. The data of the microarray have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus and are accessible through GEO series accession number GSE23902.

Real-time RT-PCR—Total RNA was treated with DNase I (Ambion, Austin, TX). cDNA synthesis from 1 μg of total RNA was performed using the SuperScript first-strand synthesis system for RT-PCR (Invitrogen). Real-time RT-PCR was performed using the ABI Prism 7900 sequence detection system (Applied Biosystems, Foster City, CA) with Power SYBR Green PCR Master Mix (Applied Biosystems) following a standard protocol recommended by the manufacturers. Primers used in this study were shown in supplemental Table S2. Relative gene expression was calculated after normalization to β-actin 5C.

Level of Reactive Oxygen Species—Adult flies were homogenized, and protein concentration was determined using a Bio-Rad protein assay kit. The level of ROS was determined using 5(6)-chloromethyl-2′, 7′-dichlorodihydrofluorescein diacetate acetyl ester (Invitrogen) as described previously (16, 17). In brief, 5 μM 5(6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate acetyl ester (in dimethyl sulfoxide) was incubated with 100 μl of total fly homogenate, and fluorescence was measured using a microplate reader at an excitation wavelength of 485 nm and an emission wavelength of 535 nm. All assays were repeated at least three times.

Antioxidant Enzyme Activities and GSH:GSSG Ratio—SOD, catalase, and GST activities were determined using an SOD determination kit (Sigma), Amplex Red catalase assay kit (Invitrogen), and GST assay kit (Cayman Chemical, Ann Ar-

bor, MI), respectively, following standard protocols as recommended by the manufacturers. GSH and GSSG were measured using a glutathione assay kit (Cayman Chemical) following a protocol provided by the manufacturer. All assays were repeated at least three times.

Administration of BSO—BSO was obtained from Sigma. Administration of BSO was performed as described previously (18) with minor modifications. In brief, BSO (1 and 6 mM) solutions were prepared immediately prior to use. Dry food powder (Carolina Biological Supply Co.) was mixed at 23.5 g/100 ml of H_2O (control food) or with BSO solution (experimental food), respectively. Second-instar larvae were collected and randomly separated into control vials or experimental vials and then transferred to the 90% O_2 chamber. The eclosion rate was determined after 3 weeks by averaging six individual vials per group. The 3–5-day-old adult flies were fed 6 mM BSO for 72 h and then transferred to the 90% O_2 chamber; survival rate was determined as described above.

Statistics—Kaplan-Meier survival analysis was used to compare life span between groups; all other data were analyzed using Student's *t* test or one-way analysis of variance and graphed using GraphPad Prism 4.02 (GraphPad Software, Inc., San Diego, CA). Results are expressed as the group mean ± S.E. Differences in means were considered statistically significant when *p* < 0.05, unless stated otherwise.

RESULTS

Overexpression of Diptericin Increases Hyperoxia Tolerance—Through experimental selection over many generations, our laboratory has previously successfully generated a *Drosophila* strain that can tolerate extremely high oxygen environments (*i.e.* 90% O_2). We have also shown that this level of hyperoxia is lethal to naive nonexposed flies. Microarray analysis has shown us additionally that the AMP family of genes (*e.g. Dpt*) is significantly up-regulated in these flies and that this up-regulation is not due to the presence of microbes (15). Here, using a *UAS/Gal4* system, we overexpressed *Dpt* by crossing *da-Gal4* with *UAS-Dpt* flies and then exposed the progeny to hyperoxia with 90% O_2. The percent eclosion rate was determined after 3 weeks in the hyperoxia chamber. As shown in Fig. 1*A*, we found that ~50% of the pupae of the *da-Gal4* × *UAS-Dpt* progeny (*da-Gal4>UAS-Dpt*) were able to eclose in 90% O_2, which was significantly higher than the *da-Gal4* (no survival) and *UAS-Dpt* (26%, *p* < 0.05) control flies, suggesting that overexpression of *Dpt* plays a critical role in the survival of hyperoxia. Furthermore, our previous data have shown that flies with decreased *Dpt* expression using RNA interference cannot eclose in 90% O_2 compared with an eclosion rate of ~10% in control flies (15), again supporting the idea that overexpression of *Dpt* contributes to the survival in hyperoxia. The life span of adult flies in hyperoxia was also determined. The *da-Gal4>UAS-Dpt* flies had an increase of ~25% in median and maximal life span over *UAS-Dpt* and *da-Gal4* control flies (*p* < 0.01) (Fig. 1*B*).

Overexpression of Diptericin Changes Gene Expression Profiles—To investigate the potential mechanisms of enhanced survival in hyperoxia observed in the *da-Gal4>UAS-Dpt* flies, we used Affymetrix microarrays to compare the
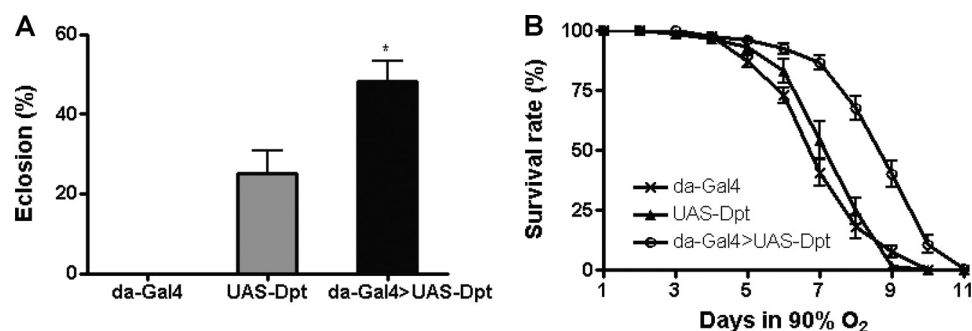
FIGURE 1. **Enhanced survival in hyperoxia.** *A*, embryos were cultured in normoxia for 48 h and then transferred to the 90% O_2 chamber; the percent eclosion was determined after 3 weeks. Flies with *Dpt* overexpression have a significantly higher eclosion rate than control flies ($p < 0.05$). *B*, the 3–5-day-old adult flies were exposed to 90% O_2, and the survival rate was scored every day. Flies with *Dpt* overexpression have a much better survival rate than control flies, a 25% increase in median and maximal life span, respectively. The Kaplan-Meier survival analysis revealed statistical significance among three groups ($p < 0.01$). *, $p < 0.05$.
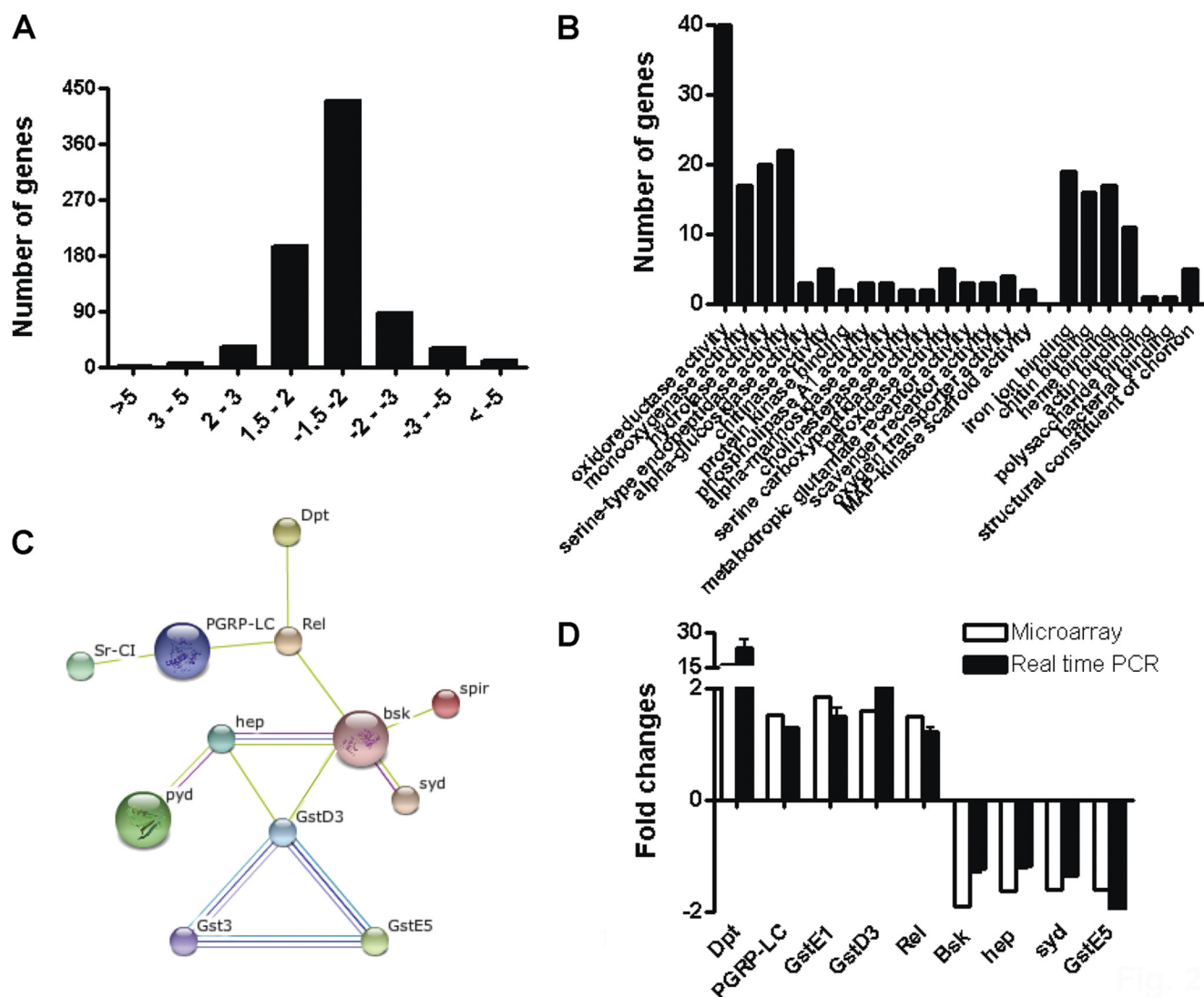

FIGURE 2. **Gene expression profile changes in flies with *Diptericin* overexpression.** *A*, the number of genes that were significantly altered in the microarrays was summarized based on their -fold changes. *B*, significantly altered biological processes in the microarrays were determined by MAPPFinder and GenMAPP 2, and data were summarized. *C*, the STRING Database predicted that *Dpt* has potential interactions with several other genes. Alternations of some genes in this network were validated using real-time PCR, and the results are summarized in *D*.

gene expression profiles between *da-Gal4>UAS-Dpt* and *UAS-Dpt* flies, with the *da-Gal4* flies included as an additional control in other assays. Remarkably, we identified 794 genes that were significantly altered in *da-Gal4>UAS-Dpt*

flies, of which 235 genes were up-regulated and 559 genes were down-regulated (Fig. 2*A* and supplemental Table S1). Using MAPPFinder in conjunction with GenMAPP, we found that genes with several biological processes, including peroxi-
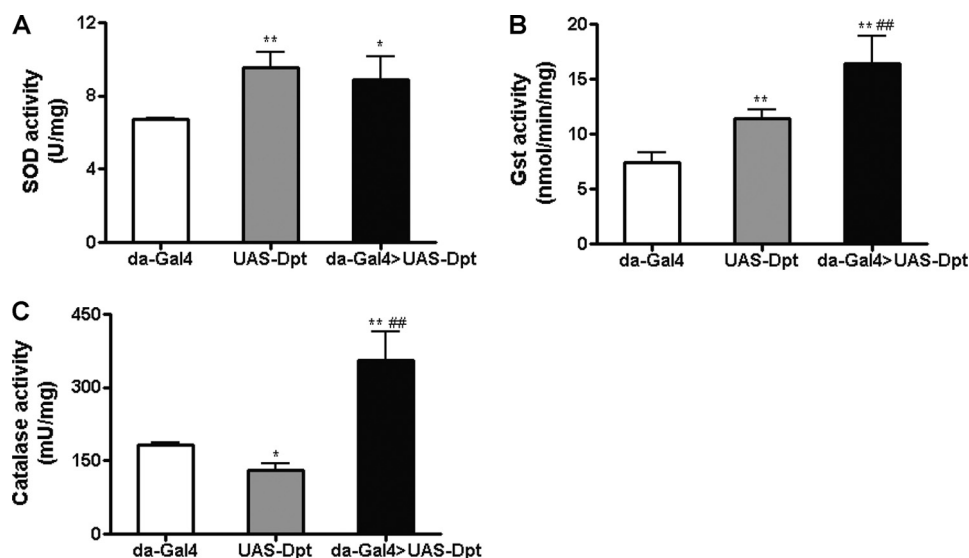
FIGURE 3. **Antioxidant enzyme activities in flies with *Diptericin* overexpression.** SOD activity (*A*), GST activity (*B*), and catalase activity (*C*) were compared among three groups. Flies with *Dpt* overexpression have significantly higher SOD, GST, and catalase activity ($p < 0.05$) than control flies, except that SOD activity is similar between *UAS-Dpt* flies and *da-Gal4>UAS-Dpt* flies. *, $p < 0.05$; **, $p < 0.01$ compared with *da-Gal4* flies; ##, $p < 0.01$ compared with *UAS-Dpt* flies.

dase activity, α-glycosidase activity, scavenger receptor activity, and MAPK scaffold activity, were significantly altered in flies with *Dpt* overexpression compared with the *UAS-Dpt* flies alone. The biological process-based categorization of significantly altered genes is summarized in Fig. 2*B*.

To determine which gene changes played a crucial role in hyperoxia tolerance and might contribute to survival in 90% O_2, we focused on all genes that were associated with significantly altered biological processes and investigated their potential network connections to *Dpt* using the STRING Database. The STRING Database, which contains known and predicted physical and functional protein-protein interactions (19), predicted that *Dpt* has interactions with several other genes, including *relish* (*rel*), *PGRP-LC*, *hemipterous* (*hep*), *basket* (*bsk*), *sunday driver* (*syd*), *spire* (*spir*), *tamou* (*pyd*), *glutathione S-transferase D3* (*GstD3*), *GstE1*, and *GstE5* (Fig. 2*C*). Alterations of gene expression in this network was validated using real-time PCR; the results were consistent with those determined by the microarrays (Fig. 2*D*).

In this network, *Gst* genes were particularly interesting because GSTs are a superfamily of multifunctional enzymes that have been implicated in a range of physiological roles such as signal transduction, cell differentiation, and apoptosis (20), as well as in the detoxification of various hydrophobic endogenous and xenobiotic compounds (21). Under conditions of oxidative stress, GSTs have glutathione peroxidase activity and protect against electrophiles and oxidative stress by altering cellular glutathione levels (21). Because of a lack of functional glutathione peroxidase enzymes in *Drosophila*, flies rely instead on the activity of the more general detoxification enzymes, GSTs, to carry out a peroxidase function (22, 23). The up-regulation of genes encoding these antioxidant enzymes (*i.e. GstD3* and *GstE1*, as well as *thioredoxin*, *CG15116*, and *peroxiredoxin 2540*) observed in our microarray analysis led us to hypothesize that flies with *Dpt* overexpression might have higher antioxidant activity and are better able to main-

tain redox homeostasis under hyperoxia. To test this hypothesis, we measured the enzyme activities of GST, as well as SOD and catalase. As shown in Fig. 3*A*, flies with *Dpt* overexpression had a significantly higher SOD activity than *da-Gal4* control flies ($p < 0.05$) but a similar activity as *UAS-Dpt* flies. The catalase and GST activity levels were significantly increased in the *da-Gal4>UAS-Dpt* flies compared with those in the *da-Gal4* and *UAS-Dpt* controls ($p < 0.05$) (Fig. 3, *B* and *C*), indicating that higher antioxidant activities observed in flies with *Dpt* overexpression may potentially contribute to the enhanced survival in hyperoxia.

Sustained Redox Homeostasis in Flies with Diptericin Overexpression—Hyperoxia treatment causes an increase in the mitochondrial ROS generation rate (24, 25) and accelerates the accrual of macromolecular oxidative damage to tissues (26, 27). An enhanced ROS level *in vivo* causes oxidation of GSH into GSSG by direct interaction with ROS or by being a substrate for the enzymatic elimination of peroxides (28–30). Therefore, a glutathione redox state, represented by the GSH:GSSG ratio, is widely used as a surrogate for determining the direction of the shift in the level of oxidative stress in tissues (28, 31, 32). Although it is not clear how flies with *Dpt* overexpression increase *Gst* gene expression and enzyme activities, our data led us to hypothesize that hyperoxia might not induce ROS accumulation or have less of an effect on redox homeostasis in these *Dpt* overexpression flies compared with controls. Thus, we determined the level of ROS and the GSH:GSSG ratio in these flies in normoxia and hyperoxia, respectively. Three days of hyperoxia treatment were chosen because 1) hyperoxia-induced oxidative damage such as increased protein carbonyl content and alteration of GSH and GSSG can be clearly observed in flies after 2.5 or 3 days in 100% O_2 (33, 34), and 2) after 3 days of hyperoxia, flies start to die rapidly. The level of ROS was measured using 5(6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate acetyl ester, a fluorescence indicator that measures ROS, including
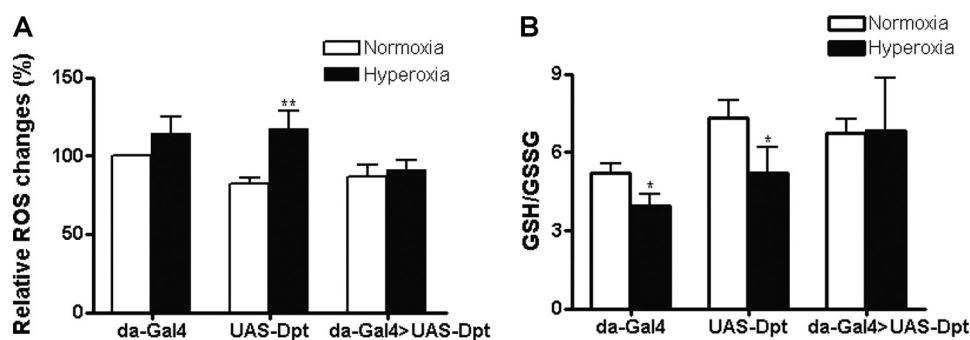
FIGURE 4. **Redox homeostasis in flies with *Diptericin* overexpression.** *A*, the level of ROS was determined by using 5(6)-chloromethyl-2′,7′-dichlorodihy-drofluorescein diacetate acetyl ester in normoxia and hyperoxia, respectively. An increased ROS level was observed in control flies ($p < 0.05$) after 3 days in hyperoxia but not in the flies with *Dpt* overexpression. *B*, a decreased GSH:GSSG ratio was observed in control flies ($p < 0.05$) but not in flies with *Dpt* over-expression. *, $p < 0.05$; **, $p < 0.01$ compared with normoxia.
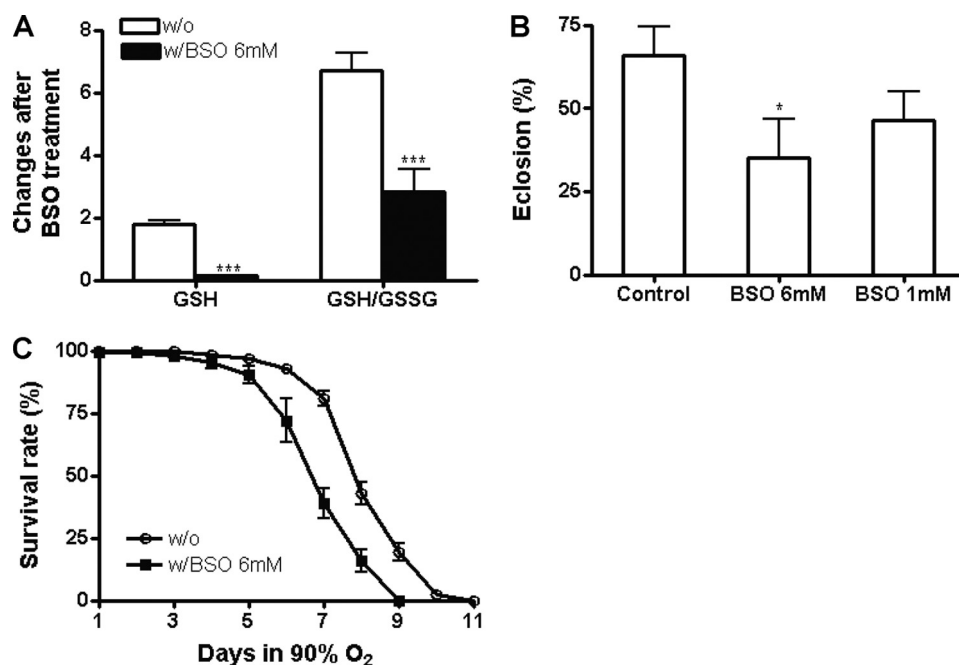

FIGURE 5. **Effect of BSO on survival in hyperoxia.** *A*, the GSH level and the GSH:GSSG ratio were compared before and after 6 mM BSO treatment in flies with *Dpt* overexpression. Both the GSH level and the GSH:GSSG ratio were significantly reduced by BSO treatment compared with the control levels ($p < 0.001$). *B*, second-instar larvae were collected, transferred to vials with or without BSO, and then exposed to 90% O$_2$. 65% of pupae from *Dpt* overexpression vials were able to eclose in 90% O$_2$ in contrast to 46 and 35% of those fed 1 mM BSO ($p = 0.07$) and 6 mM BSO ($p < 0.05$), respectively. *C*, the 3–5-day-old flies were fed 6 mM BSO food or control food for 72 h and then exposed to 90% O$_2$. The life span was significantly decreased in flies that were fed 6 mM BSO compared with flies that were not fed BSO ($p < 0.01$). *, $p < 0.05$; ***, $p < 0.0001$.

hydrogen peroxide, hydroxyl radical, peroxyl radical, and per-oxynitrite anion (35). Our data show that the level of ROS was significantly increased in *UAS-Dpt* flies after hyperoxia ($p < 0.01$) (Fig. 4*A*), and a trend toward an increase in the level of ROS was also observed in *da-Gal4* flies. However, an increased ROS level was not observed in *da-Gal4>UAS-Dpt* flies ($p > 0.05$) (Fig. 4*A*). Furthermore, the concentrations of GSH and GSSG were determined in whole-body homogenates. In normoxia, the GSH:GSSG ratio was similar among groups ($p > 0.05$) (Fig. 4*B*). However, the GSH:GSSG ratio was significantly decreased in the *UAS-Dpt* and *da-Gal4* flies following hyperoxia treatment ($p < 0.05$) (Fig. 4*B*), but no significant difference was observed in the *da-Gal4>UAS-Dpt* flies before or after hyperoxia ($p > 0.05$) (Fig. 4*B*). These data demonstrate that the glutathione redox homeostasis is maintained more efficiently in the flies with *Dpt* overexpression.

Role of Redox Homeostasis in Hyperoxia Tolerance—To further confirm whether the well maintained redox homeostasis plays an important role in hyperoxia survival, we fed flies with BSO to alter cellular glutathione levels and examined their survival in hyperoxia. BSO is a specific γ-glutamyl-cysteine synthetase inhibitor that blocks the rate-limiting step of GSH biosynthesis and depletes the intracellular GSH pool in both cultured cells and whole animals (18, 36, 37). We consistently observed that 6 mM BSO significantly reduced the GSH level and the GSH:GSSG ratio by 90 and 60%, respectively, compared with the control level ($p < 0.001$) (Fig. 5*A*). In addition, administration of BSO has been shown to decrease the activities of SOD, catalase, and glutathione peroxidase (38) and to increase ROS formation and trigger apoptosis (39). Here, after 6 mM BSO treatment, we found that 65% of pupae from *da-Gal4>UAS-Dpt* vials were able to eclose in

90% O_2 in contrast to 46 and 35% of those fed 1 mM BSO ($p = 0.07$) and 6 mM BSO ($p < 0.05$), respectively (Fig. 5*B*). The life span was also significantly decreased in adult flies after feeding 6 mM BSO ($p < 0.01$) (Fig. 5*C*).

DISCUSSION

In this study, we demonstrated that flies with *Dpt* overexpression resist hyperoxia-induced oxidative stress by increasing antioxidant enzyme activities and preventing an increase in ROS levels following hyperoxia treatment. We have further confirmed that enhanced survival observed in these flies is related to redox homeostasis with the use of BSO, a chemical that depletes the GSH pool and inhibits antioxidant enzyme activities.

It is commonly accepted that microbial infections induce a rapid and transient synthesis of AMPs in *Drosophila* and that overexpression of a single AMP restores wild-type resistance to infection in immunodeficient *Drosophila* mutants (40). In this study, we report that flies with *Dpt* overexpression survive much better than control flies, as evidenced by a significantly higher eclosion rate and longer life span in 90% O_2. These results were interesting but not surprising because many recent studies have demonstrated that, besides their antimicrobial function, AMPs have multiple roles, including inflammation, proliferation, wound healing, chemotaxis, and antiapoptosis (41–45). For instance, cecropin A and B, other AMP genes in *Drosophila*, have been shown 1) to significantly inhibit tumor cell proliferation and DNA synthesis while sparing benign fibroblast cells (46) and 2) to increase the survival time of mice bearing murine ascitic colon adenocarcinoma cells (47). Papo and Shai (48) also proposed that AMPs, which are positively charged molecules, may "recognize" negatively charged targets such as cancer cells and bacteria. Therefore, we can raise the possibility here that cationic AMPs such as *Dpt* may trap or "scavenge" free radical anions and attenuate oxygen toxicity because of their surface charge (49, 50). In addition, PR-39, a cathelicidin with antimicrobial properties, attenuates the apoptotic response of apoptosis-inducing drugs such as etoposide, bleomycin, *tert*-butylhydroperoxide, and 2-deoxy-D-ribose (51), suggesting that AMPs might act by limiting cell death and promoting cell survival under certain pathological conditions.

Through a microarray analysis, we have shown that many genes were significantly altered in the flies with *Dpt* overexpression compared with the control flies. The STRING Database analysis revealed that *Gst* genes were potentially linked to *Dpt* through several other genes or pathways. The gene changes within this network were consistent with data published previously (52, 53). For instance, *relish* activation has been linked to proteasomal degradation of *TAK1*, which leads to rapid termination of JNK signaling (52). *hep* and *bsk* have been shown to reciprocally inhibit *GstD3* activity by 50–80% *in vitro* (53). Consistent with this scenario, we observed decreased JNK signaling and increased GST activity in the flies with *Dpt* overexpression and confirmed these changes by real-time PCR and enzyme activity assays, respectively. GST has been shown to play a role in antioxidant defense and has glutathione reductase activity in *Drosophila* (20, 22, 54). In

particular, *GstD3*, which was up-regulated in the flies with *Dpt* overexpression, has also been shown to respond to dietary H_2O_2, and both *GstD3* and *GstE1* can metabolize 4-hydroxynonenal, a highly toxic aldehyde produced by lipid peroxidation in cells in response to oxidative stress (54, 55). Overexpression of classical antioxidant enzymes such as glutathione reductase, manganous SOD, catalase, and GST have been shown to increase the resistance to hyperoxia-induced oxidative stress (33, 56–60) and to attenuate cell death and aging (61, 62). As shown in this study, we have provided evidence that up-regulated antioxidant enzyme activities indeed contribute to the survival under oxidative stress. Interestingly, we noticed that *UAS-Dpt* flies have a slightly higher SOD and GST activity but a lower catalase activity than *da-Gal4* flies; thus, we speculated that the difference in antioxidant enzyme activities between *da-Gal4* and *UAS-Dpt* might explain the difference in hyperoxia survival.

Previous studies have shown that hyperoxia induces excessive ROS production, an increase in protein carbonyl accumulation and lipid peroxidation, a decrease in antioxidant enzyme activities, and a disruption of glutathione redox homeostasis (10, 11, 63). For instance, Nagato *et al.* (63) found that hyperoxia (100% O_2 for 90 min) induced a decrease in antioxidant enzyme activities, including catalase, SOD, and glutathione peroxidase; a decrease in GSH; an increase in GSSG; and a decrease in the GSH:GSSG ratio in Wistar rats. Here, we have observed that hyperoxia induced an increase in ROS levels and a decrease in the GSH:GSSG ratio in *UAS-Dpt* flies but not in flies with *Dpt* overexpression, indicating that *Dpt* directly or indirectly (through its effect on gene expression) plays an important role in survival in hyperoxia.

BSO induces oxidative stress by irreversibly inhibiting γ-glutamylcysteine synthetase, an essential enzyme for the synthesis of glutathione, and causing a rapid loss of intercellular GSH (64). Administration of BSO has been shown 1) to reduce the level of GSH and GST by 20 or 50% in *Drosophila* after feeding with 1 or 6 mM BSO, respectively, compared with the corresponding controls (18) and 2) to decrease the activities of SOD, catalase, and glutathione peroxidase (38) and to increase the sensitivity to γ-radiation and hyperoxia-induced oxidative stress (18, 65). The idea behind the use of BSO is that if redox homoeostasis in *da-Gal4>UAS-Dpt* flies played a critical role in the survival to hyperoxia, depletion of the glutathione level and a decrease in antioxidant defense by BSO would decrease their survival in hyperoxia. A significantly decreased eclosion rate and life span were observed in flies after feeding 6 mM BSO, suggesting that balanced antioxidant defense is a key factor in determining survival under hyperoxia. Although we have shown that 6 mM BSO reduced the GSH level by 90% and the GSH:GSSG ratio by 60%, the survival of *da-Gal4>UAS-Dpt* flies in hyperoxia was not abolished, suggesting that additional mechanisms must contribute to the survival in hyperoxia.

Although we have demonstrated that maintained redox homeostasis is critical in hyperoxia tolerance, we do not exclude other possible mechanisms that may contribute to the survival in the flies with *Dpt* overexpression. For instance, several JNK pathway-related genes such as *hep* and *bsk* were

significantly altered in these flies. Decreased JNK signaling has been shown to attenuate hyperoxia-induced oxidative injury. For example, by suppressing JNK signal pathways, neuropeptide substance P can promote type II alveolar epithelial cell proliferation, inhibit apoptosis after hyperoxia exposure, and attenuate hyperoxia-induced oxidative stress damage (66). Furthermore, Carnesecchi *et al.* (67) reported that hyperoxia led to phosphorylation of JNK and ERK, two MAPKs involved in cell death signaling. In contrast, hyperoxia-induced lung injury was significantly prevented in NOX1-deficient mice, in which JNK phosphorylation was blunted and ERK phosphorylation was decreased.

In summary, we have shown that flies with *Dpt* overexpression had a significantly higher eclosion rate in hyperoxia compared with control flies. The enhanced survival observed in these flies was the result, at least in part, of increasing antioxidant enzyme activities, preventing an increase in the ROS level, and maintaining GSH redox homeostasis after hyperoxia. By feeding BSO to impair antioxidant defense, we further confirmed an important role of redox homeostasis in hyperoxia-induced oxidative stresses.

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